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Co-Activation of Antagonistic Motoneurons as a Mechanism of High-Speed Hydraulic Inflation of Prey Capture Appendages in the Pteropod Mollusk *Clione limacina*

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Abstract. The predatory pteropod mollusk *Clione limacina* catches its prey by using specialized oral appendages called buccal cones. Eversion and elongation of buccal cones is a hydraulic phenomenon. In the cerebral ganglia, two groups of motoneurons have been identified that underlie functionally opposite movements of buccal cones: extrusion and retraction. We suggest that the remarkably rapid inflation of buccal cones (50 ms) is achieved through initial co-activation of antagonistic neurons, which presumably produces high pressure in the head hemocoel prior to buccal cone extrusion. The subsequent sudden inhibition of retractor motoneuron activity results in a very rapid and powerful inflation of the buccal cones. Cerebral interneurons that evoke co-activation are described.

Introduction

Feeding behavior of the pteropod mollusk *Clione limacina* has been described in several studies (Wagner, 1885; Conover and Lalli, 1972; Litvinova and Orlovsky, 1985; Lalli and Gilmer, 1989; Hermans and Satterlie, 1992). *Clione* is a predatory carnivore that feeds only on actively swimming pteropods of the genus *Limacina* and has highly specialized structures for their capture. To seize the prey, *Clione* rapidly everts six oral appendages, called buccal cones, which then become tentacle-like and grasp the *Limacina* shell, holding it during the subsequent phases of feeding (Fig. 1). The eversion and elongation of buccal cones is a hydraulic phenomenon and is accomplished by squeezing hemocoelic fluid into the central cavities of the cones. Buccal cone extrusion is a remarkably rapid reaction and occurs within 50–70 ms (Hermans and Satterlie, 1992). Such speed is not typical for hydrostatic movements in mollusks. The rapid extrusion of the *Clione* buccal cones is thus interesting from mechanical and neurobiological points of view.

Two groups of motoneurons that control the prey-capture movements of buccal cones have been identified in the cerebral ganglia of *Clione* (Norekian and Satterlie, 1993). The first group comprises a number of electrically coupled, normally silent cells called **A** motoneurons, whose activation induces opening of oral skin folds and extrusion of buccal cones. The second group of motoneurons consists of several spontaneously active cells, called **B** motoneurons, whose firing underlies retraction of buccal cones. Constant and stable spontaneous spike activity in **B** neurons maintains buccal cones in the permanently retracted position. When active, **A** neurons produce strong inhibitory inputs to **B** neurons, which terminate **B** neuron firing (Fig. 2A). Obtained data, however, revealed that **A**-to-**B** inhibition is not monosynaptic, and a single spike in an **A** motoneuron was usually ineffective in producing an inhibitory postsynaptic potential in **B** neurons (Norekian and Satterlie, 1993; Fig. 2A).

As previously mentioned, protraction of buccal cones is accomplished by squeezing hemocoelic fluid from the head hemocoel into the central cavities of the buccal cones. The speed at which this occurs depends upon the speed of synchronous activation of different muscle groups involved in buccal cone extrusion and the time required to move the hemocoelic fluid into the cone cavities. One

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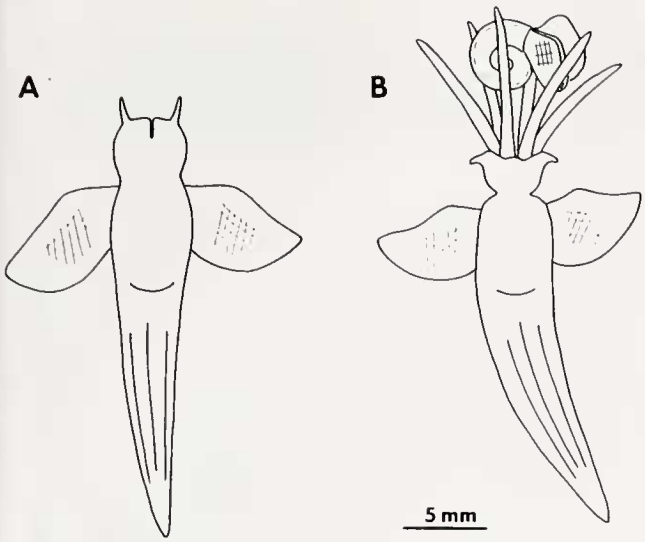

Figure 1. (A) Nonfeeding *Clione limacina* with buccal cones withdrawn inside the head and covered by skin folds (lips). (B) *Clione* with extruded buccal cones capturing the prey, *Limacina helicina*.

possible way to achieve this transfer rapidly is to increase pressure in the head hemocoel significantly prior to buccal cone extrusion. The fact that **A**-to-**B** inhibition is not monosynaptic, and **A** and **B** motoneurons, which underlie antagonistic movements of buccal cones, can be synchronously active, provides a physiological basis for this phenomenon.

Materials and Methods

Experiments were carried out at Friday Harbor Laboratories, University of Washington, in the summer and at Arizona State University in the winter and spring. Adult specimens of *Clione limacina*, 1–3 cm body length, were collected and held in large beakers of seawater at 5–10°C.

Electrophysiological experiments were performed on preparations consisting of the head, central nervous system, and wings. All nerves running from the central ganglia to the head and to the wings were intact, but body nerves were cut. The preparations were tightly pinned to a Sylgard-coated Petri dish with cactus spines (*Opuntia* sp.). Prior to recording, ganglia were desheathed by bathing the preparation in a 1 mg/ml solution of protease (Sigma type XIV) for approximately 5 min, followed by a 30-min wash.

For intracellular recordings, glass microelectrodes were filled with 2 *M* potassium acetate and had resistances of 10–20 MΩ. Electrophysiological signals were amplified, displayed, and recorded using conventional techniques. Intracellular stimulation was provided via amplifier bridge circuits. For morphological investigation of recorded neurons, a 5% solution of 5(6)-carboxyfluorescein (Sigma) prepared in 2 *M* potassium acetate was iontophoresed via the recording electrodes with 0.5–10 nA negative current pulses for 20–30 min. Resistances of the electrodes were 20–30 MΩ. Injected cells were observed and photographed live in the recording dish with an incident-light fluorescent microscope (Nikon).

Tactile stimulation of the head and lips was provided by a thin polymeric filament, 0.2 mm in diameter. Fifty-six preparations were used in the experiments.

Results

Co-activation of antagonistic motoneurons

During simultaneous recordings of **A** and **B** neuron activities, common excitatory inputs were recorded in both types of neurons (Fig. 2B, C). Some of these depolarizing inputs were subthreshold for **A** neuron activity and produced only an increase in **B** neuron firing. Others evoked a few spikes in **A** neurons, but were insufficient to initiate **A**-to-**B** neuron inhibition, resulting in a short period of co-activation of both types of neurons (Fig. 2B). This kind of neuron activity did not produce notable behavioral responses in the preparations. Buccal cones remained withdrawn inside the head, covered by skin folds (lips). When spontaneous, common inputs were of greater strength and sufficient to initiate prolonged **A** neuron bursting, they produced co-activation of **A** and **B** neurons followed by sudden inhibition of **B** neurons and continuation of **A** neuron activity (Fig. 2C). Such strong bursting activity of **A** neurons, which resulted in inhibition of **B** neurons, was always correlated with a strong behavioral reaction of the preparation—opening of the oral skin folds (lips) and partial extrusion of buccal cones (schematic drawings in Fig. 2C). In intact animals, buccal cones become tentacular, extending approximately one-half of a body length (Fig. 1B). The necessary compromise of the fluid skeleton prevented full extension of buccal cones in dissected preparations. Nevertheless, all behavioral events underlying buccal cone extrusion could be observed; these include opening of the oral skin folds, general contraction of the head wall muscles, contraction of buccal cone circular muscles, and eventually a partial expansion of the buccal cones (schematic drawings in Fig. 2C).

Initial co-activation of **A** and **B** neurons, when followed by inhibition of **B** neurons and continuation of **A** neuron activity, typically lasted for around 1 s. The result of **A** and **B** neuron co-activation would be the contraction of head wall and neck musculature (**A** motoneuron activity) with maintained retraction of the buccal cones (**B** motoneuron activity), that would lead to a significant increase of fluid pressure in the head hemocoel of intact *Clione*. The subsequent sudden inhibition of **B** neuron activity, with continued activation of **A** neurons, would result in a very rapid and powerful inflation of the buccal cones.

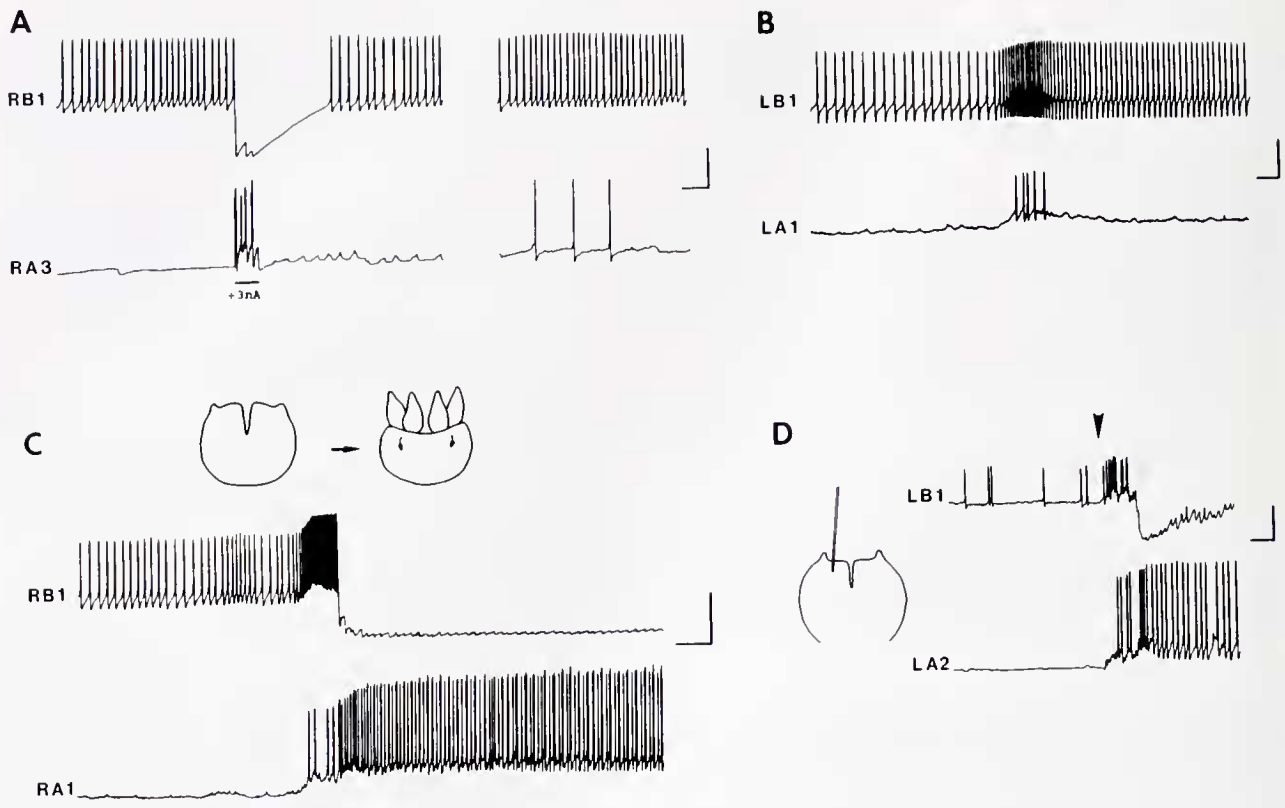

Figure 2. (A) Activation of **A** neurons produced high amplitude inhibitory potentials in **B** neurons. However, **A**-to-**B** inhibition is polysynaptic, and individual spikes in **A** neurons were usually insufficient to produce inhibitory potentials in **B** neurons. (B) Spontaneous, common excitatory inputs were recorded in both **A** and **B** neurons. (C) When common excitatory inputs were strong enough to produce powerful and prolonged spike activity in **A** neurons, they evoked initial co-activation of both types of neurons followed by **B** neuron inhibition with continuation of **A** neuron firing. This type of neuron activity was closely correlated with the behavioral response of the head as shown by schematic drawings. In silent preparations, buccal cones are withdrawn inside the head and covered by oral skin folds. When bursts of spikes appeared in **A** neurons and inhibited **B** neurons, the skin folds moved laterally and the buccal cones were partially extruded. (D) Tactile stimulation of the anterior region of the head, including lips (shown by schematic drawing), produced common excitatory inputs in both **A** and **B** neurons. These inputs were sometimes strong enough to initiate co-activation of both types of neurons followed by inhibition of **B** neurons with continuation of **A** neuron firing. The moment of stimulation is shown by arrow. Scale bars = 15 mV, 2 s.

Such common excitatory inputs, which are capable of producing co-activation of **A** and **B** neurons, can arise from contact with prey. In several studies, it was shown that the capture of *Limacina* by *Clione* was initiated by direct contact with the prey (Conover and Lalli, 1972; Lalli and Gilmer, 1989; Litvinova and Orlovsky, 1985). Tactile stimulus from the prey thus appears to play an important role in initiating extrusion of buccal cones. Tactile stimulation of the anterior part of the head and the lips of *Clione* produced excitatory inputs to both **A** and **B** neurons (Fig. 2D). These common excitatory inputs were sometimes strong enough to initiate co-activation of **A** and **B** neurons followed by inhibition of **B** neuron firing and continuation of **A** neuron activity (Fig. 2D). This kind of neuron activity resulted in the opening of the skin folds and the partial extrusion of the buccal cones. Therefore, common excitatory inputs to **A** and **B** neurons arising from contact with prey can provide a contributory mechanism underlying the high speed of hydrostatic extrusion of buccal cones during prey capture in *Clione*.

Interneurons producing co-activation of A and B motoneurons

One pair of neurons that produced synchronous excitatory inputs to both **A** and **B** motoneurons was identified in the cerebral ganglia. Due to their *coordinating* influence on **A** and **B** motoneuron activities, these were designated C_{AB} neurons. Cell bodies of C_{AB} neurons were small, 15–30 μm in diameter, and were situated on the anterior

margin of the cerebral ganglia between head nerves N1 and N2 (Fig. 3A). Neurons were silent or had low frequency (0.5 Hz) spontaneous spike activity, with membrane potentials between −55 and −60 mV. Activation of a recorded C_{AB} neuron by injecting depolarizing current pulses resulted in the appearance of excitatory postsynaptic potentials in **A** motoneurons and biphasic excitatory-inhibitory responses in **B** motoneurons (Fig. 3B). The primary response of **B** motoneurons was a burst of fast excitatory postsynaptic potentials that produced a short burst of increased spike activity (1-s duration, spike frequency up to 10 Hz). The spike burst was followed by a slow hyperpolarizing wave that terminated **B** neuron firing for 10–30 s after C_{AB} neuron activation. In **A** motoneurons, C_{AB} neurons produced fast excitatory postsynaptic potentials that were able to induce **A** neuron spike activity (Fig. 3B). Sometimes the spike activity in **A** motoneurons was not strong enough to activate **A**-to-**B** inhibition (Fig. 3B), but at other times it produced fast inhibitory potentials in **B** motoneurons, masking the slow inhibitory response induced directly by C_{AB} neurons. Thus, the typical response to C_{AB} neuron activation was initial, brief co-activation of **A** and **B** motoneurons followed by prolonged **B** neuron inhibition, induced by fast **A**-to-**B** inhibitory postsynaptic potentials, slow hyperpolarizing waves, or both.

Connections between C_{AB} neurons and **A** and **B** motoneurons appear to be monosynaptic. Each spike in a C_{AB} neuron produced an individual excitatory postsynaptic potential in a recorded **A** motoneuron with a stable, short latency of 2.5 ms (Fig. 4A). Similarly, each C_{AB} neuron spike produced an individual excitatory postsynaptic potential in a recorded **B** motoneuron with a stable latency of 4 ms (Fig. 4B). Moreover, spike durations in C_{AB} neurons, which varied during bursting activity, were reflected in the amplitude of excitatory postsynaptic potentials in **A** and **B** neurons. The second and third spikes in the C_{AB} neuron were wider than the first spike (Fig. 4A), and as a consequence, the second and third postsynaptic potentials in the **A** neuron had higher amplitudes than the first. In addition, a high Mg^{++}/high Ca^{++} seawater (110 m*M* $MgCl_2$, 25 m*M* $CaCl_2$) did not block postsynaptic potentials in **A** and **B** motoneurons induced by C_{AB} neurons (Fig. 4C). In high Mg^{++}/high Ca^{++} solution, **B** motoneurons demonstrated both components of the biphasic synaptic response, and slow hyperpolarization was detected even after two to three C_{AB} neuron spikes. Biphasic monosynaptic contacts can be explained by the existence of two types of receptors on the membrane of **B** motoneurons for the same transmitter, or by the release of co-transmitters by the C_{AB} neurons.

Activation of a single C_{AB} neuron produced similar responses in ipsilateral and contralateral **A** and **B** motoneurons (Fig. 4C). Carboxyfluorescein injections of C_{AB} neurons revealed that each neuron had many small, thin processes around the cell body in the ipsilateral ganglion and one large axon running through the paracerebral connective to the contralateral cerebral ganglion (Fig. 5). This paracerebral axon of C_{AB} neuron appears to underlie the observed contralateral connections.

Contralateral C_{AB} neurons were electrically coupled. Electrotonic coupling was demonstrated by applying depolarizing or hyperpolarizing square current pulses to one neuron and recording similar but attenuated responses simultaneously in the contralateral neuron (Fig. 6A). Electrical coupling was sufficient to produce 1:1 spike ac-

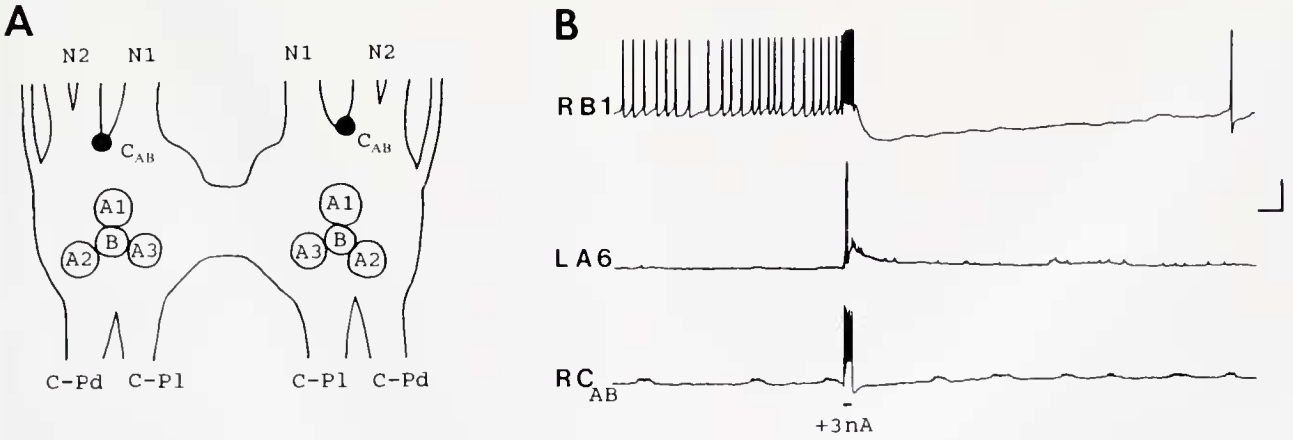

Figure 3. (A) Position of C_{AB} neuron somata in the cerebral ganglia; N1 and N2—head nerves; C-Pd and C-Pl—cerebro-pedal and cerebro-pleural connectives; some of the **A** and **B** motoneurons are also shown. (B) Effect of RC_{AB} neuron stimulation on the activities of **LA6** and **RB1** motoneurons. Notice the initial, short co-activation of **A** and **B** motoneurons and subsequent appearance of a slow hyperpolarizing wave in **B** motoneurons. Scale bars = 15 mV, 2 s.

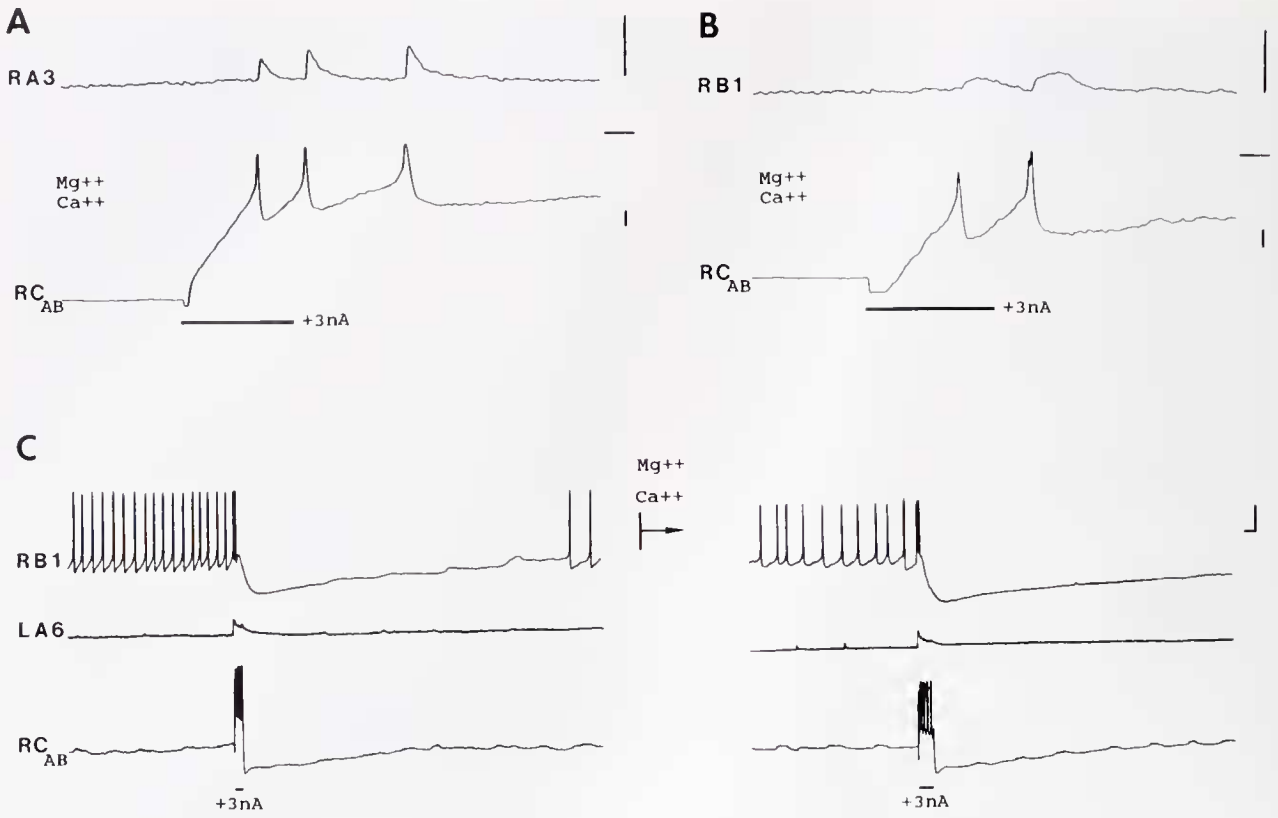

Figure 4. (A, B) Each spike in the C_{AB} neuron produced individual excitatory postsynaptic potentials in **A** and **B** neurons with stable short latencies even in high Mg^{++}/high Ca^{++} solution. Scale bars = 10 mV, 50 ms. (C) High Mg^{++}/high Ca^{++} saline did not influence any of the types of postsynaptic potentials in **A** and **B** motoneurons after C_{AB} neuron activation. Scale bars = 15 mV, 1 s.

tivity in C_{AB} neurons (Fig. 6A), suggesting that electrical coupling plays an important role in synchronizing activities of contralateral C_{AB} neurons.

B motoneurons did not induce responses in cerebral C_{AB} neurons, whereas strong activation of **A** motoneurons produced inhibitory inputs in C_{AB} neurons (Fig. 6B). This connection was not monosynaptic, because individual spikes in **A** neurons did not produce individual inhibitory postsynaptic potentials in C_{AB} neurons, and only strong burst activity was able to induce C_{AB} neuron inhibition. These inhibitory inputs presumably serve as negative feedback, preventing reverberation of impulses between the two groups of neurons.

As previously mentioned, mechanical contact with prey produces significant sensory inputs that initiate buccal cone extrusion. Tactile stimulation of the anterior region of the head, including the lips, produced excitatory inputs in C_{AB} neurons that were able to initiate spike activity in C_{AB} neurons (Fig. 7). Thus, C_{AB} neurons, which produce co-activation of **A** and **B** motoneurons, are included in the neuron pathway that underlies the rapid extrusion of buccal cones initiated by mechanical inputs from the prey.

Discussion

Expansion of the buccal cones of *Clione* is a hydraulic phenomenon accomplished through squeezing of hemocoelic fluid from the head hemocoel into the hemocoelic cavities of the buccal cones. Ordinarily, such hydraulic inflation of tentacular structures is a relatively slow process, as seen in the erection of tentacles in pulmonates, which is dependent on hydrostatic pressure in the cephalopedal sinus (Dale, 1973). In *Clione*, however, buccal cone expansion is extremely fast, occurring in 50 to 70 ms (Hermans and Satterlie, 1992).

There are only a few examples of such fast reactions in mollusks. The best known example, which is behaviorally analogous to *Clione* buccal cone expansion, is the prey capture reaction of squid tentacles, which elongate fully in 15 to 30 ms (Keir, 1985). However, the mechanisms by which the buccal cones and squid tentacles move to grasp their prey are quite distinct. Cephalopod tentacles are muscular hydrostats that do not rely on volume changes in fluid-filled cavities (Kier, 1985). Their elongation is a purely muscular phenomenon, so the speed of

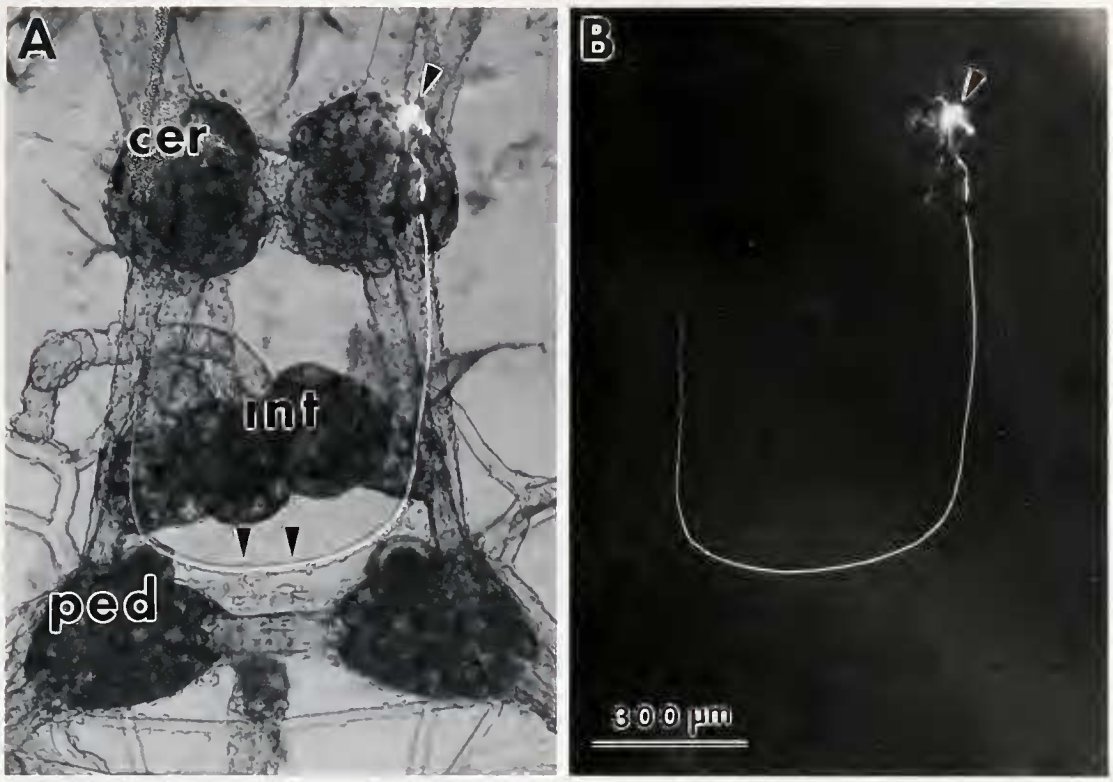

Figure 5. (A, B) Morphology of C_{AB} neurons revealed by carboxyfluorescein injections. A single arrow shows the position of the cell body of neuron RC_{AB}. Double arrows show the paracerebral connective; cer—cerebral ganglion, ped—pedal ganglion, int—intestinal gangha.

their reaction depends only on the speed of muscle contraction. This raises the interesting question of how the *Clione* buccal cones, which are hydrostatic structures, expand at a rate that is within the range of muscular elongation. We propose here that co-activation of functionally reciprocal **A** and **B** motoneuron groups, which evoke protraction and retraction of buccal cones respectively, is critical for their rapid and powerful extrusion.

The time of *Clione* buccal cone expansion depends on the time required for synchronizing the activities of all muscles involved in producing this reaction, and on the time required for moving hemocoelic fluid into the cone cavities. The first factor, synchronization, is a problem because buccal cone expansion requires the contraction of many muscle groups, including head wall muscles, neck muscles, circular muscles of buccal cones, and muscles opening the skin folds. Twenty-six **A** motoneurons whose activity evokes contraction of these muscles have been identified in the cerebral ganglia of *Clione* (Norekian and Satterlie, 1993). Synchronized activity of all **A** neurons is required for initiating buccal cone extrusion. One second of co-activation observed in **A** and **B** motoneurons prior to buccal cone inflation would give time for synchronization of activities of all **A** neurons and all muscle groups participating in this reaction.

The second problem is the necessity to eject hemocoelic fluid into cone cavities as rapidly and forcefully as possible. Two factors appear to contribute to resolving this problem. First, Hermans and Satterlie (1992) demonstrated that expansion of buccal cones is associated with a 23% reduction in head diameter and a distinct circular constriction in the neck region, without any notable contractions of body wall muscles. Lalli (1967) described a muscular diaphragm that surrounds the anterior aorta in the neck region and appears to separate head and body hemocoels. Closure of this diaphragm would allow isolation of the head hemocoel so that rapid contractions of the head and neck muscles could be translated rapidly and efficiently into unidirectional fluid movements into the buccal cones. Second, a method of greatly increasing pressure in the head hemocoel immediately prior to buccal cone inflation would greatly increase the rate of buccal cone inflation. Co-activation of antagonistic **A** and **B** motoneurons, which evokes protraction and retraction of buccal cones respectively, appears to be a mechanism capable of significantly increasing the pressure inside the head prior to buccal cone extrusion. Contraction of all muscles participating in buccal cone extrusion tends to force hemocoelic fluid into the cone cavities while simultaneous contraction of retractor muscles opposes this fluid movement, pre-

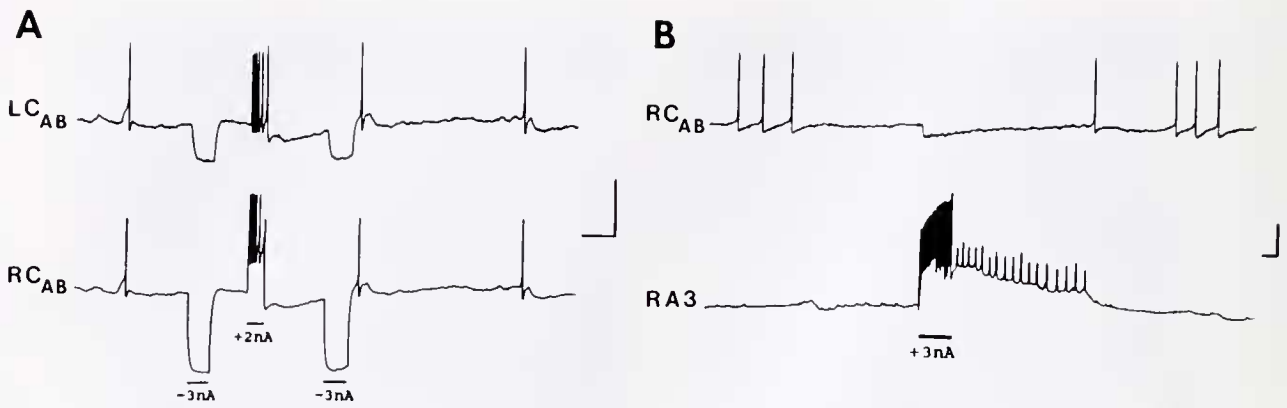

Figure 6. (A) Contralateral C_{AB} neurons are electrically coupled as demonstrated by applying depolarizing or hyperpolarizing square current pulses to one neuron and recording similar responses simultaneously in the contralateral neuron. Scale bars = 15 mV, 2 s. (B) Activation of A neurons produced inhibitory inputs in C_{AB} neurons. Scale bars = 10 mV, 1 s.

sumably producing a *significant* increase in blood pressure inside the head. Subsequent sudden inhibition of the retractor neurons and, therefore, relaxation of retractor muscles would lead to a very forceful and rapid ejection of hemocoelic fluid from the head into cone cavities and to inflation of buccal cones. Behavioral observations with high-speed cinematographic analyses show an initial bulging of the head immediately prior to buccal cone extrusion (Hermans and Satterlie, 1992), supporting the hypothesis of co-activation of antagonistic muscle groups.

Similar mechanisms underlying powerful and rapid reactions have been found in other animals. In the locust, energy for the jump is stored in the elastic elements of the leg during a short period of co-contraction of hindleg extensor and flexor muscles. A sudden inhibition of flexor activity transfers the stored energy to rapid extension movements of the hindlegs (Heitler and Burrows, 1977). For an analogy based on fluid pressure, we can look at the mammalian heart. During the brief isometric contraction phase of the ventricles, when both sets of ven-

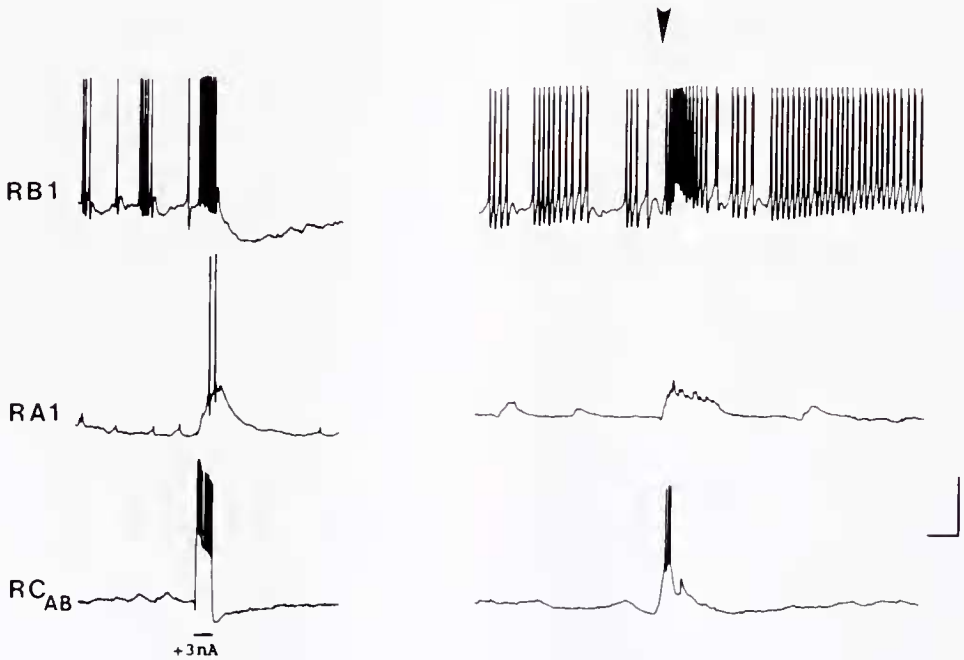

Figure 7. C_{AB} neurons were identified based on the reactions they produced in **A** and **B** neurons. Tactile stimulation of the anterior region of the head, including lips, produced excitatory inputs in all three types of neurons including neuron C_{AB}. The moment of tactile stimulation is shown by the arrow. Scale bars = 20 mV, 2 s.

tricular valves are closed, fluid pressure builds rapidly, thus allowing blood to be more forcefully ejected after opening of the semilunar valves.

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